

Application of hybrid LRR technique to protein crystallization

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LRR family proteins play important roles in a variety of physiological processes. To facilitate their production and crystallization, we have invented a novel method termed “Hybrid LRR Technique”. Using this technique, the first crystal structures of three TLR family proteins could be determined. In this review, design principles and application of the technique to protein crystallization will be summarized. For crystallization of TLRs, hagfish VLR receptors were chosen as the fusion partners and the TLR and the VLR fragments were fused at the conserved LxxLxLxxN motif to minimize local structural incompatibility. TLR-VLR hybridization did not disturb structures and functions of the target TLR proteins. The Hybrid LRR Technique is a general technique that can be applied to structural studies of other LRR proteins. It may also have broader application in biochemical and medical application of LRR proteins by modifying them without compromising their structural integrity. [BMB reports 2008; 41(5): 353-357]

Structural characteristics of the LRR family

LRR family is a large protein family with ~300 genes in human and ~6000 entries in the database (1-5). They are involved in a wide variety of physiological processes mostly by interacting with other proteins. LRR family proteins share conserved tandem modular motives and horseshoe like three dimensional structures (6). Common structural pattern can be recognized among ~50 reported LRR structures (1). All LRR family proteins contain multiple copies of LRR modules that are protected by special LRRNT and LRRCT modules at their termini. Individual LRR modules have 20-30 amino acid residues with a highly conserved LxxLxLxxN motif (1, 4, 7). Side chains of the conserved leucines that can be replaced by other hydrophobic residues point inside forming the extended hydrophobic core. Asparagines in the motif make continuous hydrogen bonds with backbone carbonyls of neighboring β strands throughout the entire protein (8-10). This extended hydrogen bond network is called “asparagine ladder”. The con-

served asparagines in the network can be replaced by other residues, cysteines, threonines or serine that are capable of hydrogen bond formation (1, 7, 11). The variable “X” residues in the LRR motives are hydrophilic and exposed to the concave surfaces of the horseshoe like structure. They are not involved in the structural stabilization and can be replaced by other hydrophilic residues without deterioration of protein stability. Therefore, in the LRR modules, the conserved residues provide rigid structural framework and the variable residues are available for function.

LRR family can be classified into seven subfamilies. Each subfamily has characteristic module length and secondary structural pattern (1, 12). For example, “typical” subfamily members have 24 amino acids in the module and contain 3₁₀ helices and loops in the convex area of the horseshoe like structure. On the other hand, RI-like subfamily proteins have 28 amino acids and contain alpha helices in the convex area (2, 4, 7). The convex structure play important role in determining overall radius of the protein because alpha helices require more space than 3₁₀ helices (13). Therefore, “typical” subfamily proteins show larger radius than RI-like subfamily proteins. Vast majority of the LRR family proteins faithfully adopt the common structural pattern of the family but several new LRR structures show unexpected large deviations from the common pattern. CD14, TLR1-4 structures show irregular structural pattern in the convex area which seems essential for their ligand binding function (14-16). Structure of the auxin receptor has sharp structural transition in the central β sheet (17).

LRR family proteins are involved in a large variety of physiological functions including, immune response, cell cycle regulation, enzyme regulation, etc (18). Most of them are mediated by protein-protein interaction. The concave surface appears to be the most frequent area for protein interaction; Ribonuclease inhibitor, Glycoprotein Ib, VLR, Internalin interact with their protein ligands in the concave surface (19-22). TLRs 1, 2, 4 and CD14 are exceptions for this common structural pattern (14-16). Part of their convex surface contains a crevice that is connected to a large internal pocket necessary for binding of lipid containing ligands.

Design principles of Hybrid LRR technique

Many LRR family proteins are difficult to produce in quantities enough for crystallization. Even if they could be produced in large quantities, they often could not be crystallized for x-ray

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diffraction studies. To overcome these fundamental problems in structural biology, we invented a novel method termed “Hybrid LRR Technique” (15, 16). Using this technique, we successfully determined structures of seven TLR-VLR hybrid proteins last year, demonstrating its usefulness in structural biology. We have shown that our LRR fusion strategy does not interfere with natural structures and functions of the parental proteins.

To generate hybrid proteins in high success rate, we need to choose structurally compatible fusion partners and fusion strategy that does not generate atomic collisions at the fusion sites. Variable Lymphocyte Receptors (VLRs) were chosen as the fusion partners for TLR research. They are adaptive immune receptors recently discovered in jawless fishes, lamprey and hagfish (23). Unlike mammalian immunoglobulins, their sequences and structures belong to LRR family (24). Diversity of VLR clones is generated by somatic recombination of several hundreds of germ line genes encoding LRR modules (25). Hagfish VLRs have two subfamilies VLRA and VLRB that have 3-6 LRR modules. Theoretically more than 10^{16} clones can be generated by recombination. For TLR research, VLRs were chosen as fusion partners because they are (i) easy to produce, easy to crystallize. (ii) They all adopt standard LRR structures. (iii) Therefore, the most compatible VLR can be select among the almost limitless clone pool. Although VLRs were shown to be very successful fusion partners for Hybrid LRR Technique, other LRR proteins should also be vigorously tested. For non typical subfamily, fusion partners other than VLR may be better because VLR belongs to the “typical” subfamily of LRR (26).

As the fusion strategy, TLR and VLR fragments are fused at the most conserved “LxxLxLxxN” sites because they are easy to be identified from amino acid sequences and because they always form β strands that can always be superimposable among different LRR structures (Fig. 1) (4, 7). It is very important to keep the relative positions of the conserved leucines and asparagines because single amino acid shift may reorient the side chains to completely opposite direction. Nearly unlimited combination of TLR and VLR can be generated by changing fusion sites in TLR, fusion sites in VLR and identity of VLR clone.

Even if we fuse two LRR fragments at the conserved “LxxLxLxxN” site, structural incompatibility of VLR with TLR at the three dimensional fold is inevitable in some cases because structure of convex part of TLR is unpredictable. As described in the next paragraph, success rate of our Hybrid LRR Technique was shown to be about 50% using TLR1, TLR2, TLR4 and VLR (15, 16). Modeling study suggests that half of the unsuccessful hybrids have either structural collision of TLR and VLR in the convex area or exposure of hydrophobic core in the convex surface of the fusion site. This kind of problem cannot be predicted and experimental selection of compatible fusion partner from diverse library should be included in the fusion strategy. Nevertheless, 50% success rate is unexpectedly high and hybrid proteins suitable for crystallographic or other study can be selected among relatively small library of hybrid proteins.

Application to TLR4 crystallization

Toll-like receptors (TLRs) play a central role in mammalian innate immunity (6, 27). They are expressed on plasma membrane or intracellular organelles and recognize conserved structural patterns in microbial products that are generally absent from host molecules (28, 29). Until now, ten TLRs are identified in human and six subfamilies have been defined based on sequence homologies (1, 12). All TLR proteins contain a single transmembrane domain, a conserved intracellular signaling domain named TIR and an extracellular ligand binding domain composed of LRR modules (28, 29). Among TLR family members, TLR1, 2 and 4 have been studied using the Hybrid LRR Technique (15, 16).

TLR4 associates with MD-2 and recognizes LPS from Gram negative bacteria (30, 31). MD-2 belongs to ML family and contains a large lipid binding pocket interacting with LPS (32). LPS is a major component of outer membrane of Gram negative bacteria and is one of the strongest inducer of innate immune response (33). Immune response by LPS is mediated by the hydrophobic lipid A region. The core and O-antigen polysaccharide chain is dispensable for immune response and TLR4-MD-2 binding. Different bacterial species produce lipid A containing different number and chain lengths of lipid chains. LPS is extracted from the bacterial membrane and transferred to the TLR4-MD-2 complex by two accessory proteins, LPS-binding Protein (LBP) and CD14 (34).

Since its discovery about 10 years ago (27), TLR4 eluded worldwide effort of crystallization. It was difficult to produce in a large quantity and could not be crystallized by conventional crystallization techniques. To facilitate soluble expression and crystallization of the TLR4-MD-2 complex with bound ligands, TLR4-VLR hybrids were generated by the Hybrid LRR Technique (15). Seven out of 15 designed hybrids yielded near-

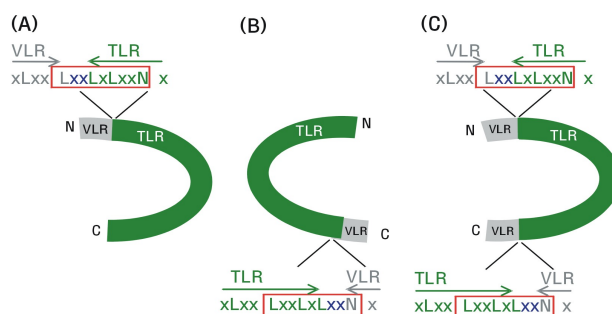


Fig. 1. Strategy to fuse the TLR and VLR clone using Hybrid LRR technique. Several fusion strategies of TLR and VLR fragments in N-terminal (A), C-terminal (B) and both termini (C) are represented. TLR and hagfish VLR fragments in the LRR hybrids are coloured in green and gray, respectively. Conserved sequences at the fusion boundaries are written in red boxes. The sequences written in blue are non-native sequences from the cloning sites.

ly three times more than native TLR4 and three of them were successfully crystallized although none of MD-2 and LPS exists. This means the fusion with TLR4 and VLR fragment is sufficient driving force for crystallization without the stabilization due to interaction with other protein or ligand. Among three hybrids, two can bind to MD-2 and LPS and can be dimerized by LPS binding, demonstrating that functional integrity of the protein is not compromised by the Hybrid LRR Technique. The remaining one hybrid clone did not bind to MD-2 or LPS because it did not have regions responsible for the binding.

Structural analysis of the TLR4 hybrids demonstrates that the Hybrid LRR Technique did not disturb native structures of VLR and TLR4. Since structures of VLR clones used for hybrid formation have been already determined, we could superimpose it with those of the VLR modules in the hybrid proteins. The backbone atoms of the native and hybrid proteins had practically identical structures, with an average C α rms difference of 0.3–0.4 Å after superimposition (Table 1) (15). We also compared the structures of the overlapping regions of the three TLR hybrids. The overlapping regions have near identical backbone structures with average C α rms differences of 0.39 Å and 0.28 Å, respectively. These minor changes are unlikely to be due to the structural incompatibility of TLR4 with VLR. TLR3 structures determined by two different laboratories are compared as a control, and their average Ca rms difference was 1.1 Å (15). The structural homology is not restricted to the backbone atoms; even at the fusion site the side chains have similar conformations, apart from the long and flexible ones. The unexpected structural rigidity of the LRR modules is surprising but understandable since the concave β strand region of LRR proteins is an unusually rigid structural frame. From the analysis described above, we concluded that the Hybrid LRR technique did not cause any substantial structural changes either to VLR or to TLR4.

Application to TLR1 and 2 crystallization

TLR2 forms a heterodimer with TLR1 or TLR6 and mediates physiological recognition of bacterial lipoproteins and lip-

oteichoic acids (35, 36). Bacterial lipoproteins are anchored to bacterial membrane through three lipid chains covalently attached to the conserved N-terminal cysteine (37). Lipoteichoic acids are amphipathic molecules found in the membrane of Gram positive bacteria and induce strong proinflammatory signals in macrophages (38–40).

The TLR1 and 2 complex was difficult to produce and to crystallize. Therefore Hybrid LRR Technique was used for large scale production and crystallization of the TLR1-TLR2 complex (16). Similar with TLR4-VLR hybrids, fragments of TLR and a VLR clone were fused at their "LxxLxLxxN" sites while the relative positions of the conserved leucines and asparagines were strictly preserved (Fig. 1). 33 out of 62 hybrids tested could be produced as soluble proteins. The expression amount of the TLR1- and TLR2-VLR hybrids was increased with similar rate of TLR4 hybrids. Later modeling study suggests that most of the hybrids that failed to produce soluble proteins are either to form atomic collisions or to expose hydrophobic cores at the fusion sites. Some of the TLR1-VLR and TLR2-VLR hybrids could form a heterodimer by binding of ligand, demonstrating their function integrity. Among the soluble hybrids, the ligand induced heterodimer of the TLR1-VLR and TLR2-VLR hybrids was successfully crystallized for structure analysis (Fig. 2).

The Hybrid LRR technique was crucial to the structure determination of TLR1-TLR2 complex as well (16). The full length ectodomain of TLR1 could not be produced in sufficient quantity for crystallization, and the full length ectodomain of TLR2 could be produced but could not be crystallized. The hybrid proteins contributed to structural determination as well. The structures of the longer TLR hybrids could not be directly solved using any known LRR family proteins as search probes for the molecular replacement calculation. However, the shorter TLR2 hybrid had sufficient structural homology with the Nogo receptor, and its structure could be solved by the molecular replacement technique (8). The refined structure of the shorter hybrid in turn served as a good search probe for the longer TLR1 or TLR2-VLR hybrids.

Similar with TLR4-VLR hybrids, we concluded that TLR-VLR

Table 1. C α rms differences of full-length VLRB.61 and the VLR fragments in the TLR4-VLR hybrids

TLR4-VLR hybrid	VLR residues in the hybrid	Ca rmsd (Å)
VT3	24C ~ 82L	0.37
TV3	228L ~ 302P	0.45
TV8	530N ~ 596P	0.44

Backbone atoms of the full-length VLRB.61 (PDB accession 2O6R) and the VLR portions of hybrids are superimposed (9, 15). The PDB coordinates of the VT3, TV3 and TV8 are 2z66, 2z62 and 2z63, respectively. The construct names of TLR4-VLR hybrids are written as proposed by Kim and Park *et al* (15).

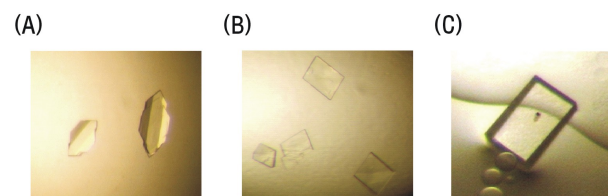


Fig. 2. Crystals of TLR2 and lipopeptide and its complex with TLR1. Successfully crystallized TLR-VLR hybrids using Hybrid LRR technique are shown. All crystals of TLR-VLR hybrids were obtained at 23°C by the hanging-drop vapor diffusion method (16). Crystals of mouse TLR2-VLR hybrid complexed with its lipopeptide ligand Pam₂CSK₄ (A) and Pam₃CSK₄ (B) were produced in condition of 0.2 M ammonium sulfate, 0.1 M Tris-HCl pH 8.5 and 34% PEG1000. Human TLR1-TLR2 hybrid bound to Pam₃CSK₄ (C) was crystallized using a solution containing 0.2 M sodium citrate, 20% PEG3350.

fusion did not interrupt native structures of TLR or VLR. First, the structures of the VLR fragments in all four hybrids and the native VLR are essentially identical with backbone rms differences of 0.4-0.9 Å (16). Not only the backbone atoms but also the majority of side chains adopts the same conformations in the native and the hybrid VLR proteins, even at the fusion boundaries. Second, the overlapping regions of the TLR2-VLR hybrids are practically identical in two TLR2-VLR hybrids. Among seven hybrids of TLR1, TLR2 and TLR4-VLR hybrids, none of them show any sign of structural alterations induced by fusion. This strongly supports our proposal that the Hybrid LRR Technique does not induce any significant structural changes to LRR fragments in the hybrids.

Conclusion and future prospects

We have shown that the Hybrid LRR technique is useful for structural studies of LRR family proteins in general. There are more than 6000 LRR proteins in the database. They are involved in a large variety of physiological processes that are often critical in human pathology. Therefore structural studies of these LRR proteins using the Hybrid LRR Technique will make significant contribute to their biological and medical research. The Hybrid LRR technique may also have broader applications other than crystallization. It can be used to reduce the size of proteins to the minimum required for function. It can also be applied to generate multifunctional LRR proteins.

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